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Graft-vs.-host and graft-vs.-leukemia reactions after delayed infusions of donor T-subsets

Bryon D. Johnson, Emily E. Becker, Robert L. Truitt

Department of Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin

Offprint requests: Bryon D. Johnson, PhD, Department of Pediatrics, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226; bjohnson@mcw.edu

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ABSTRACT

Infusions of donor leukocytes have been given to allogeneic bone marrow recipients after transplant to treat leukemia relapse. Treatment with these delayed infusions of donor cells has been called delayed or donor leukocyte infusion (DLI). While graft-vs.-host disease (GVHD) has typically been less severe than expected after DLI, it still remains a significant risk factor. Recently, we used a full major histocompatibility complex (MHC)-mismatched model (C57BL/6 into AKR) to determine how increased immunogenetic disparity affects GVH and graft-vs.-leukemia (GVL) reactions after DLI. In contrast to an MHC-matched model (B10.BR into AKR), GVHD was still observed when MHC-mismatched donor T cells were infused 3 weeks posttransplant. Limiting dilution analysis was used to determine the frequency of alloreactive cytotoxic T lymphocytes (CTL) and interleukin (IL)-2–secreting T helper cells in the spleens of MHC-mismatched recipients 7 days after DLI treatment. GVHD correlated with elevated frequencies of alloreactive T-helper cells. One strategy for reducing the severity of GVHD after DLI is the selective administration of CD4 or CD8 T-subsets. Delayed infusion of purified T-subsets 3 weeks posttransplant resulted in significantly less GVHD than infusion of a mixture of the two subsets. No GVH-associated mortality was observed after DLI with purified donor $CD4^+$ T cells. In GVL studies, MHC-mismatched $CD8^+$ T cells were the most potent antitumor effectors against an acute T cell leukemia. The GVL effect of MHC-mismatched T-subsets was compared with that of MHC-matched subsets. When naive MHC-matched cells were given as DLI, depletion of either T-subset eliminated the GVL effect. $CD8^+$ T cells from MHC-matched donors primed against host alloantigens, however, mediated a CD4 (T-helper)–independent GVL reaction. Together, these results suggest that administration of T-subsets can significantly reduce GVHD after DLI without loss of the beneficial GVL effect.

KEY WORDS:

Donor leukocyte infusion • T-subset • Graft-vs.-leukemia • Graft-vs.-host disease

INTRODUCTION

Allogeneic BMT is being used to treat leukemia that is refractory to conventional therapy. Problems associated with this treatment still remain, however, including GVHD and leukemia relapse. GVHD can be avoided if donor T cells in the marrow graft are removed, but elimination of the T cells increases the risk for leukemia relapse [1]. The antitumor benefit provided by the donor T cells is known as the graft-vs.-leukemia (GVL) effect. The ongoing challenge for researchers and clinicians is to find ways of harnessing the beneficial GVL effect of allogeneic BMT while avoiding life-threatening GVHD.

If a leukemia patient relapses after an allogeneic BMT, treatment options are limited and include additional chemotherapy, a second marrow transplant, or cytokine therapy [2]. Unfortunately, these treatments have not been highly successful and are often associated with unacceptable toxicities. Cellular immunotherapy, given as infusions of lymphocytes from the original BM donor, is currently being used worldwide to successfully treat posttransplant leukemia relapse. This treatment, often referred to as delayed or donor lymphocyte infusion (DLI), has been particularly successful for treating CML relapse. Success rates as high as 80% have been reported [3,4]. DLI has been less successful

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for treating acute leukemia; complete remissions have been observed in only 15–18% of treated patients [4]. The surprising aspect of DLI therapy is that relatively large numbers of donor T cells can be given with less GVHD than anticipated if similar numbers of T cells were given at the time of BMT. In fact, some patients given DLI have been successfully treated without developing GVHD [3–6]. For most patients treated with DLI, however, GVHD remains a life-threatening problem occurring in ~60% of patients [4]. A major issue for DLI is whether the incidence of GVHD can be further decreased without affecting the beneficial GVL effect. To address this issue, the GVL effector cells need to be defined and characterized.

Our laboratory has developed murine models to investigate important clinical issues regarding DLI therapy. We previously demonstrated that DLI could be given to MHC-matched murine recipients to provide a GVL effect without GVHD if the donor cell administration was delayed until at least 3 weeks posttransplant [7,8]. In this report, we investigate how increased immunogenetic disparity in an MHC-mismatched combination of murine allogeneic BMT affects GVHD after DLI. In addition, the effect of T-subset depletion on GVH and GVL reactivity after DLI was examined in MHC-mismatched and -matched models.

MATERIALS AND METHODS

Mice

AKR (H-2^k, Thy1.1), B10.BR (H-2^k, Thy1.2), and C57BL/6 (H-2^b, Thy1.2) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and cared for in the Medical College of Wisconsin Animal Resource Center (Milwaukee, WI), which is accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC).

Bone marrow transplants

AKR hosts (6–8 weeks old) were preconditioned for BMT with a lethal dose of 1100 cGy total body irradiation (TBI) from a cesium source (irradiator). BM was obtained by flushing the femurs and tibias of normal, healthy B10.BR or C57BL/6 donors. Two to four hours after irradiation, the hosts were given a single i.v. injection of $0.75–1.5\times10^7$ donor BM cells with or without admixed splenocytes or purified splenic T cells. In selected experiments, the donor BM was depleted of T cells *ex vivo* or *in vivo*. For *ex vivo* depletion, the BM cells were treated with bioreactor-derived anti-Thy1.2 monoclonal antibody (clone 30-H12; ATCC, Rockville, MD) and complement. For *in vivo* depletion, the marrow donors were injected i.p. with 75 µg anti-Thy1.2 monoclonal antibody 5 and 2 days before BM harvest. T cell depletion was confirmed by flow cytometric analysis.

For DLI experiments, donor spleen cells or purified splenic T cells were given i.v. at various times posttransplant. For T cell purification, a magnetic-activated cell separation (MACS) system (Miltenyi Biotec, Sunnyvale, CA) was used. T cells were positively selected in magnetic columns after incubation with anti-CD4, anti-CD8, or anti-Thy1.2 conjugated microbeads. The MACS microbeads are very small (~50 nm in diameter) and have not affected cell function in our studies. For depletion of CD4 or CD8 T-subsets, spleen cells were treated with anti-L3T4 (clone GK1.5; ATCC) or anti-Lyt2.2 (clone 2.43; ATCC) and complement, respectively.

For preimmunization of donors against AKR alloantigens, B10.BR mice were injected i.p. twice a week with 10^7 normal AKR splenocytes. One week after the final injection, the donors were killed and the spleens harvested for use in DLI experiments.

Leukemia

A spontaneously arising acute T cell leukemia of AKR origin was isolated from the spleen of a male AKR mouse and passaged in vivo. After passage, the leukemia (designated M2) was reisolated and frozen. Vials of the frozen stock were thawed for leukemia-challenge experiments. This strategy was used to minimize experimental variability. The leukemia cells are $CD4^+$, $Thy1.1^+$, MHC class I^+, MHC-class II^-, and variable for CD8 expression. For tumor challenge experiments, mice were injected i.v. with indicated numbers of leukemia cells.

Culture medium

The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, antibiotics, and additional amino acids [9].

Limiting dilution analysis (LDA) assays

LDA assays were used to determine T-helper cell and cytolytic T lymphocyte (CTL) precursor frequencies as previously described [8]. The spleen cells from three individual BMT chimeras were isolated and pooled. Responder T cells were enriched by positive selection using MACS with anti-Thy1.2 conjugated microbeads. To assess alloreactive T-helper cell frequencies, Thy1.2-enriched responder cells were cocultured at eight different concentrations in 16 replicate U-bottomed microwells with 2.5×10^4 irradiated (3000 cGy) AKR peritoneal exudate cells (200 µL/well total volume). After 4 days of culture in a 37°C, 10% CO_2 incubator, 150 µL of culture supernatant was removed from each well and transferred to a new microwell plate containing 4000 interleukin (IL)-2–dependent SAC 9.12 [10] indicator cells and 1 µCi [^{3}H]thymidine. The plates were cultured an additional 18–24 hours, and thymidine uptake was assessed. Spontaneous thymidine uptake was determined from the supernatants of 48 wells that had contained stimulator cells only.

To assess CTL frequencies, various numbers of Thy1.2-enriched responder cells were cocultured with 5×10^5 irradiated (3000 cGy) AKR spleen cell stimulators (16 wells per responder cell concentration). Concanavalin A (con A)–stimulated rat spleen cell conditioned medium was added to the wells at a concentration of 40% (vol/vol). The plates were cultured for 8 days. On day 8, the contents of each well was mixed, and 150 µL of cell suspension was transferred to V-bottomed microtiter plates with 50 µL [^{51}Cr]-labeled AKR con A–stimulated lymphoblast targets (5000 per well). ^{51}Cr release was assessed after a 3.5-hour incubation. Spontaneous and maximum ^{51}Cr release values were determined from 16 wells containing target cells plus stimulators only or detergent (2.5% 7× detergent; Flow Laboratories), respectively.

Individual wells in the T-helper cell and CTL assays were scored as positive when experimental values (cpm [^{3}H]thymidine uptake or percent specific ^{51}Cr release) exceeded the spontaneous control values by at least 3SD. The frequencies of alloreactive T-helper cells and CTL were calculated by χ^2 minimization as described by Taswell [11].

Statistics

Survival curves were compared using the log-rank test.

RESULTS

DLI with MHC-mismatched donor cells can be given without causing GVHD at 4 weeks posttransplant

We previously showed in MHC-matched and -haplomismatched models of allogeneic BMT that donor lymphocytes can be given to recipients 3 weeks after transplant without any clinical evidence of GVHD [7,8]. In this study, our transplant model was adapted to study the impact of full MHC disparity. AKR recipients were given a lethal dose of TBI and injected i.v. with 10^7 MHC-mismatched C57BL/6 BM cells with or without different doses of spleen cells ($0.5–3.0\times10^7$) as a source of donor T cells. Groups of mice given BM only received DLI 3 weeks after marrow transplant. The experiments were staggered so that the groups given DLI were transplanted 3 weeks before those groups given spleen cells on the day of marrow transplant. In this way, the spleen cells given to all groups were from the same pool of cells.

As shown in Fig. 1A, all groups of mice given spleen cells on the day of transplant died. They rapidly developed clinical signs of acute GVHD (diarrhea, body weight loss, and ruffled fur). When the infusion of donor spleen cells was delayed until 21 days posttransplant (Fig. 1B), significant GVH-related mortality still occurred at all doses of splenocytes administered. There was no statistically significant difference in mortality between the groups that received 1.0, 2.0, or 3.0×10^7 splenocytes (~$0.4–1.2\times10^9$/kg body weight); however, there was significantly less mortality ($p = 0.02$) in the group given 0.5×10^7 splenocytes (0.2×10^9/kg) vs. the group given 3.0×10^7 splenocytes (1.2×10^9/kg) (Fig. 1B). While significant GVHD was observed after DLI on day 21 post-BMT, all groups given DLI (with the exception of the group given 2.0×10^7 cells) had significantly better survival rates than their respective counterparts given donor spleen cells on the day of transplant ($p < 0.008$). These data indicate that GVHD occurred after DLI at 21 days posttransplant in this MHC-mismatched model, but that GVH-associated mortality was less than that observed when comparable numbers of donor cells were given at the time of marrow transplantation.

The next series of experiments was designed to investigate whether GVHD could be avoided if DLI was delayed longer than 21 days posttransplant. When infusion of 3×10^7 donor spleen cells (~1.2×10^9/kg) was delayed until 28 days posttransplant, all recipients survived to 90 days post-BMT (Fig. 2A). These animals exhibited little or no body weight loss after DLI (Fig. 2B), reflecting the absence of clinically evident GVHD. Survival and clinical appearance were identical to that of the controls given non–T-depleted BM only. In contrast, 50% of the mice given DLI 1 week earlier (day 21) died of GVHD-related complications by 90 days posttransplant (Fig. 2A). The presence of GVHD in these animals was reflected by the 15–30% body weight loss after DLI (Fig. 2B).

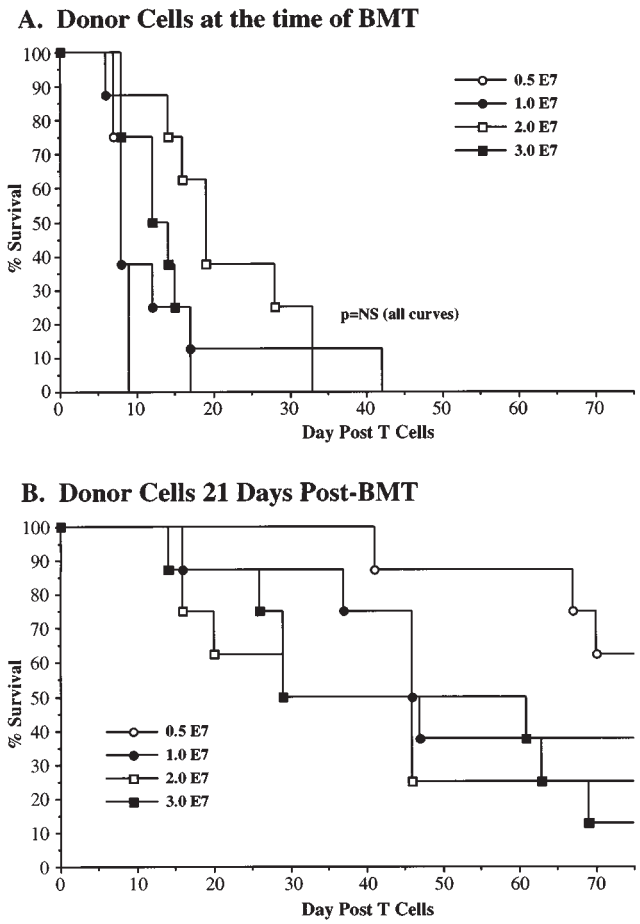

Figure 1. Lethal GVHD was not avoided when DLI was administered to MHC-mismatched chimeras 21 days posttransplant

*AKR recipients, preconditioned with lethal TBI, were injected i.v. with 10^7 non–T-depleted C57BL/6 BM cells. Doses of 0.5, 1, 2, or 3×10^7 donor spleen cells (~0.2 to 1.2×10^9/kg) were coadministered with the BM (***A***; n=8 mice/dose) or given as a single i.v. injection 21 days after BMT (***B***; n=8 mice/dose). The survival data were derived from the combination of two independent experiments. Day 0 represents the day donor spleen cells were given (either the day of BMT or 21 days post-BMT). None of the survival curves in* ***A*** *were statistically different* ($p > 0.05$).

We previously reported a correlation between low frequency of alloreactive T-helper cells and absence of GVHD [8]. In the current studies, T cells were isolated from the spleens of MHC-mismatched chimeras and tested in LDA assays to assess alloreactive T-helper cell and CTL frequencies. As shown in Table 1, three groups of AKR recipients were transplanted: *1*) a GVH-negative control group given C57BL/6 non–T-depleted BM only; *2*) an experimental group given C57BL/6 non–T-depleted BM followed by DLI with 3×10^7 donor spleen cells 21 days post-BMT; and *3*) a GVH-positive control group given 3×10^7 donor spleen cells at the time of BMT. Mice given donor spleen cells were killed 7 days after administration (i.e., either 28 or 7 days post-BMT), and the mice given BM only were killed

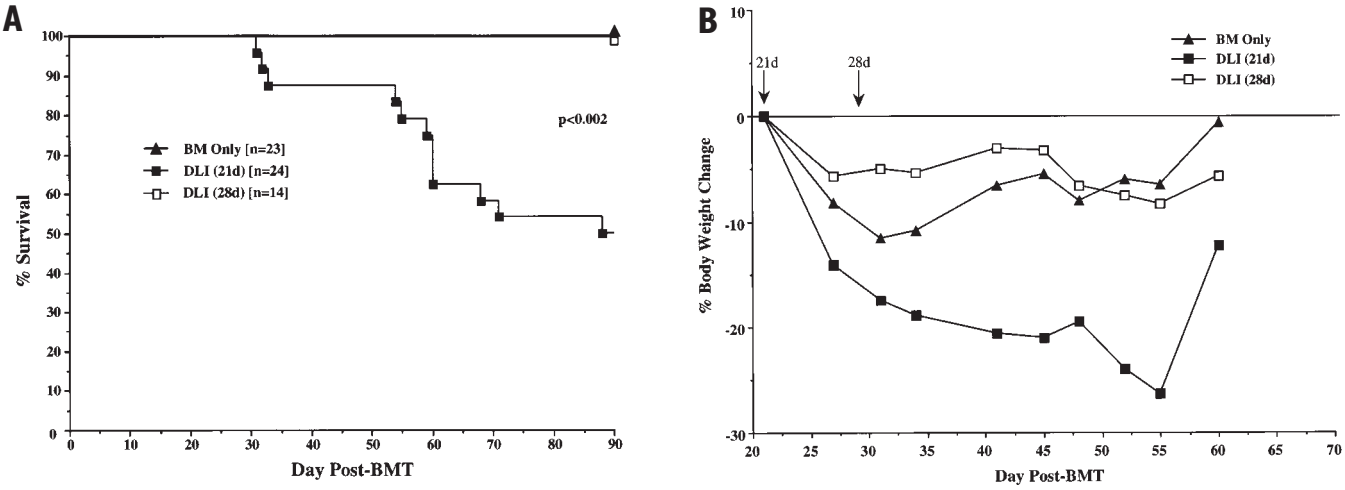

Figure 2. GVHD was avoided in MHC-mismatched chimeras if DLI administration was delayed until 28 days posttransplant

*Irradiated AKR recipients were given 10^7 non–T-depleted C57BL/6 BM cells. The recipients were then randomized to receive no further treatment (BM only) or DLI with 3×10^7 C57BL/6 donor spleen cells at 21 (21d) or 28 (28d) days post-BMT. The curves show the combined posttransplant survival data of two experiments (**A**) and the percent body weight loss or gain of recipients from one representative experiment starting at 21 days post-BMT (**B**).*

28 days post-BMT. Splenic T cells were isolated and tested in LDA assays. The frequencies of alloreactive CTL in the spleen were significantly different (nonoverlapping 95% confidence intervals) between the three groups of chimeras, the GVH-positive controls having the highest frequency and the GVH-negative controls the lowest frequency (Table 1). The frequency of alloreactive T-helper cells between the GVH-positive control group and the DLI experimental group were not significantly different; however, both groups had significantly higher T-helper frequencies than the GVH-negative control group. Since the experimental mice given DLI at 21 days post-BMT developed GVHD (Figs. 1 and 2), these results suggest a correlation between alloreactive T-helper cell frequencies and the intensity of GVHD.

$CD4^+$ or $CD8^+$ T-subsets given 21 days posttransplant do not cause GVHD

Purified $CD4^+$ and $CD8^+$ T cells were used to determine whether infusion of T-subsets could reduce the severity of GVHD after DLI at 21 days in MHC-mismatched recipients. AKR hosts were transplanted with C57BL/6 BM alone or admixed with purified T cells or T cell subsets. Transplants were staggered so that all groups received donor T cells on the same day from the same pool of cells. The T cell suspensions were analyzed by flow cytometry before infusion, so that the numbers of T-subset cells administered (3×10^6 for $CD4^+$ and 2×10^6 for $CD8^+$) could be adjusted to correspond with their respective numbers within the unseparated T cell–enriched pool. The enriched T cells were >90% pure, and the subset-enriched cells contained ≤1% contaminating cells of the opposite subset. As shown in Fig. 3, purified donor T cells and both T-subsets given on the day of BMT induced lethal GVHD. When the infusion of T cells was delayed until 21 days post-BMT, mixed T cells induced significantly less GVH-related mortality ($p = 0.038$) than when given at the time of transplant (25 vs 0% survival), but GVHD was still severe as reflected by body weight loss after infusion (Fig. 3B). DLI with T-subsets resulted in significantly less GVH-related mortality compared with DLI with mixed T cells ($p < 0.047$) (Fig. 3A). Mice given DLI with $CD4^+$ donor T cells had no deaths, regained body weight after a modest initial loss of 10% (Fig. 3B), and exhibited a healthy clinical appearance. Although 30% of the mice given DLI with $CD8^+$ donor T cells died after infusion, the survivors regained body weight after an initial loss and appeared generally healthy thereafter.

Table 1. *LDA assays to estimate the frequency of alloreactive CTL and IL-2-secreting T helper cells in the spleens of C57BL/6-AKR into chimeras 7 days post-T cells*

			CTL frequencies		T-helper frequencies	
Spleen cell source	Day postinfusion	Day post-BMT	CTL/10^6 cells	Fold increase over BM alone	$T_H/10^6$ cells	Fold Increase over BM alone
BM alone	—	28	<20		656	
BMS-30 (21d)	7	28	314	>16	1493*	2
BMS-30 (0d)	7	7	6,667	>333	1938*	3

Day postinfusion or post-BMT indicates the time when recipients were killed for LDA analysis. BM alone, C57BL/6 BM cells only; BMS-30 (21d), C57BL/6 BM cells followed 21 days later by 3×10^7 C57BL/6 spleen cells; BMS-30 (0d), C57BL/6 BM cells admixed with 3×10^7 C57BL/6 spleen cells.

**Overlapping 95% confidence intervals.*

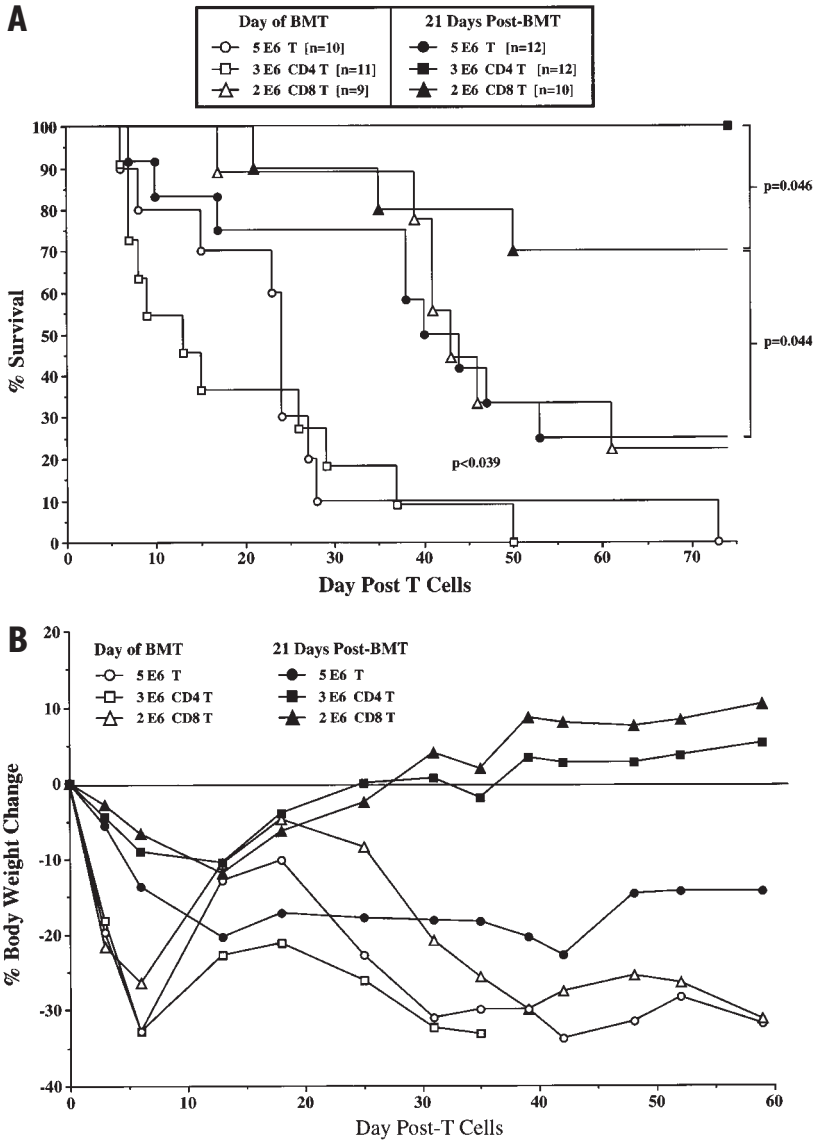

Figure 3. DLI with purified T cells resulted in significantly less severe GVHD

*Irradiated AKR recipients were given 10^7 MHC-mismatched non–T-depleted C57BL/6 BM cells. The recipients were then randomized to receive 5×10^6 purified donor T cells, 3×10^6 purified donor CD4+ cells, or 2×10^6 purified donor CD8+ T cells on the day of BMT (○, □, △) or 21 days posttransplant (●, ■, ▲). The curves show the combined survival data of two experiments (**A**) and the percent body weight loss or gain of recipients in one representative experiment (**B**; n=4–6 mice/group).*

Some survivors from the experiment shown in Fig. 3A were killed 74 days after DLI to examine spleen size and B cell content (Table 2). Splenic atrophy [12] and low B cell content [13] are long-term sequelae of GVH reactivity. Two survivors given $CD8^+$ T cells on the day of marrow transplant had small spleens (mean 1.3×10^6 cells) and low B cell content (mean 9.6%). Similarly, one survivor given DLI with mixed donor T cells had a small spleen and relatively low B cell content. In contrast, the mice given DLI with $CD4^+$ or $CD8^+$ T cells had larger spleen sizes (mean 43.1×10^6 and 50.8×10^6 cells, respectively) and higher B cell content (mean 63.8 and 65.0%). Together, these results demonstrated that DLI at 21 days with donor $CD4^+$ or $CD8^+$ T cells significantly reduced the severity of GVHD and improved immune recovery compared with DLI with unseparated T cells.

$CD8^+$ donor T cells are the principal effector cells in the GVL response induced by DLI

MHC antigens are potent tumor rejection antigens, whereas non-MHC encoded (minor) histocompatibility antigens are less potent and often require presensitization or priming of the donor to elicit a strong antitumor effect [14]. In the final series of experiments reported here, we examined the GVH reactivity of T-subsets from naive MHC-mismatched, naive MHC-matched, and primed MHC-matched donors.

Table 2. *Spleen sizes and splenic B cell content in long-term survivors of DLI with T-subsets*

Group*	Day of infusion†	n	Spleen size ($\times 10^6$)	Percent splenic B cells
$CD8^+$	0	2	1.3 ± 0.34	9.6 ± 8.1
Mixed	21	1	0.5	24.0
$CD4^+$	21	6	43.1 ± 22.6	63.8 ± 8.5
$CD8^+$	21	4	50.8 ± 4.0	65.0 ± 7.3

Viable splenic cell number was determined by visual counting and percent splenic B cells by flow cytometry. Data are averages ± SD.

**Survivors from the experiments shown in Fig. 3 were killed 74 days after T cell infusion.*

†Post-BMT.

First, $CD4^+$ and $CD8^+$ T cells were purified from the spleens of naive C57BL/6 donors and given as DLI to MHC-mismatched AKR hosts. The host mice, previously transplanted with T cell–depleted C57BL/6 BM, were randomized to be given *1*) no DLI (BM only); *2*) DLI at 28 days post-BMT with a mixture of 3×10^6 purified $CD4^+$ and 3×10^6 purified $CD8^+$ T cells; *3*) DLI with 3×10^6 purified $CD4^+$ T cells alone; or *4*) DLI with 3×10^6 purified $CD8^+$ T cells alone. DLI was given 28 days posttransplant to avoid the GVHD observed when T cells were given at an earlier time (Fig. 2). Mice within each experimental group were randomized to be challenged with 100 (Fig. 4A), 1000 (Fig. 4B) or 10,000 (Fig. 4C) AKR-M2 leukemia cells on day 35 post-BMT (7 days post-DLI). When challenged with a relatively low dose of 100 leukemia cells, all groups given DLI had a significant GVL response as indicated by increased survival over the control group given no DLI (BM only) (Fig. 4A). The mice given $CD8^+$ cells had a higher survival rate than mice given $CD4^+$ cells (56 vs. 27%), but the differ-

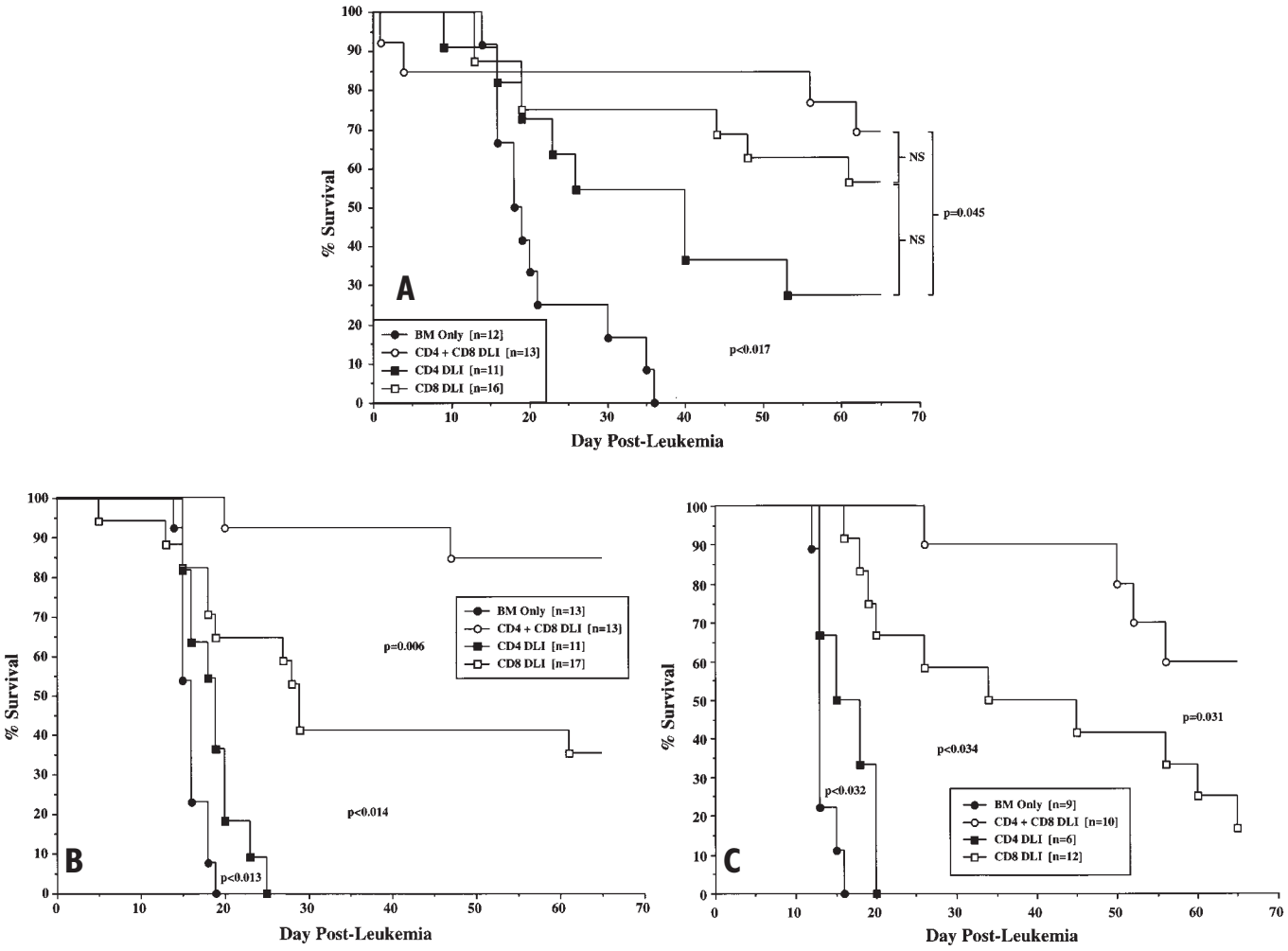

Figure 4. $CD8^+$ T cells are the predominant GVL effector population in MHC-mismatched chimeras given DLI

*Irradiated AKR recipients ere transplanted with 10^7 T-depleted C57BL/6 BM cells. Recipients were then randomized to receive no further treatment (BM only) or DLI 28 days posttransplant with 3×10^6 $CD4^+$ donor T cells, 3×10^6 $CD8^+$ cells, or a mixture of 3×10^6 $CD4^+$ and 3×10^6 $CD8^+$ cells. One week later, (35 days post-BMT), the mice were challenged with 100 (**A**), 1000 (**B**), or 10,000 (**C**) AKR-M2 leukemia cells. The survival curves in each figure were derived from the combined results of three experiments.*

Table 3. *Quantitative measure of the GVL effect in MHC-mismatched chimeras given DLI with purified T cells*

DLI*	LD_{50} dose	r^2
None	<100	
$CD4^+$	<100	
$CD8^+$	384	0.985
$CD4^+ + CD8^+$	7775	0.916

For each group, a regression line was generated by plotting log leukemia dose (100, 1000, and 10,000 cells) vs. percent death. The dose of leukemia needed to kill 50% of mice (LD_{50}) was determined from the regression line.
**Groups of mice from the experiments in Fig. 4.*

ence was not statistically significant. Survival in the CD8 DLI group was not significantly different from survival in the group given the mixture of both T subsets (56 vs. 69%). After challenge with the intermediate (1000) and high (10,000) doses of leukemia cells, only the mice given $CD8^+$ cells or the CD4/CD8 mixture showed a GVL effect as manifested by increased survival rates over the BM-only controls. At these higher doses of leukemia, mice given DLI with $CD4^+$ cells survived significantly longer than the mice given BM only, but median survival time was only increased by 3–4 days, and all eventually died.

To document whether mice in these experiments were dying of tumor progression or GVHD, they were autopsied for the presence of splenomegaly, an indicator of tumor progression in this experimental system, or splenic and thymic atrophy, which is suggestive of GVHD. All of the BM-only control mice were found to have splenomegaly at the time of death, indicating that they died of tumor progression (data not shown). In the groups of mice given DLI with the mixture of both T cell subsets, only two of the 10 deaths appeared to be due to leukemia progression, while six of the remaining eight mice had splenic and thymic atrophy. These results suggest that the majority of deaths in these experimental groups were due to GVHD rather than leukemia. In contrast, the majority of mice in the groups given DLI with $CD4^+$ or $CD8^+$ T cells appeared to die of leukemia progression. Only two of 25 deaths in $CD4^+$ DLI groups and two of 28 deaths in the $CD8^+$ DLI groups appeared be from GVHD as indicated by splenic and thymic atrophy. Taken together, these observations suggest that the majority of the deaths in these experiments, with the exception of the mice given DLI with the mixture of both T subsets, appeared to be due to leukemia progression and not GVHD.

Data from the experiments in Fig. 4 were used to quantify the GVL effect in each group of mice. The dose of leukemia needed to kill 50% of mice (LD_{50}) was calculated from regression curves generated by plotting log leukemia dose vs. percent death (Table 3). The LD_{50} values for the groups given no DLI (none) or DLI with purified $CD4^+$ cells were too low to be quantified (LD_{50} < 100 leukemia cells). DLI with $CD8^+$ cells had a quantifiably greater GVL effect (LD_{50} = 384 leukemia cells) than DLI with $CD4^+$ cells or BM only, but the LD_{50} was 20-fold higher with mixed CD4/CD8 T cells (LD_{50} = 7775 leukemia cells), indicating a synergistic (helper) effect when $CD4^+$ cells were added to the $CD8^+$ effector cells.

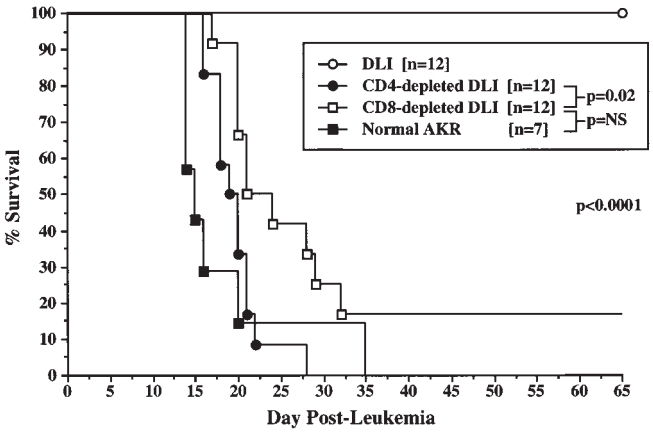

Figure 5. DLI with donor cells depleted of $CD4^+$ or $CD8^+$ T cells significantly compromised GVL reactivity in MHC-matched recipients

Irradiated AKR recipients were given 10^7 T-depleted B10.BR BM cells. The recipients were then given DLI at 21 days posttransplant with 3×10^7 B10.BR spleen cells that were untreated, CD4-depleted, or CD8-depleted. One week later (28 days post-BMT), the mice were challenged with a lethal i.v. dose of 100 AKR-M2 leukemia cells. A group of normal, nontransplanted AKR mice were injected with the leukemia as a control. The survival curves were derived from the combined results of two experiments.

Additional groups of AKR mice were transplanted with T-depleted MHC-matched B10.BR BM and randomized to receive DLI at 21 days post-BMT using *1*) B10.BR spleen cells, *2*) spleen cells depleted of $CD4^+$ T cells, or *3*) spleen cells depleted of $CD8^+$ T cells. All mice were challenged with a lethal dose of 100 AKR-M2 leukemia cells 7 days after DLI (28 days post-BMT) to assess GVL reactivity. Normal nontransplanted AKR mice were injected as leukemia controls. Mice given DLI with nondepleted donor spleen cells exhibited a strong GVL effect (100% survival; Fig. 5). These mice were followed as long as 139 days postchallenge and were without clinically evident GVHD (data not shown). Depletion of either $CD4^+$ or $CD8^+$ naive T cells significantly compromised the GVL effect (Fig. 5). Survival rates for these mice (0% for CD4-depleted and 17% for CD8-depleted) were not significantly higher than for the nontransplanted AKR leukemia control group.

Spleen cells from B10.BR donor mice preimmunized against AKR alloantigens were tested in DLI experiments to determine if primed T cells induced a stronger GVL effect. AKR mice that had been previously transplanted with T cell–depleted B10.BR BM were infused 21 days post-BMT with primed anti-host–reactive donor spleen cells (see MATERIALS AND METHODS for immunization protocol) or primed spleen cells depleted of $CD4^+$ or $CD8^+$ T-subsets. One week later, the mice were challenged with 1000 AKR-M2 leukemia cells. DLI with primed unseparated donor splenocytes elicited an effective GVL response (Fig. 6), and the mice did not develop clinically evident GVHD. In contrast to the results with naive donor cells (Fig. 5), the mice given DLI with primed CD4-depleted ($CD8^+$) donor spleen cells had a significant GVL response (Fig. 6). The survival rate for this group (75%) was not statistically different from

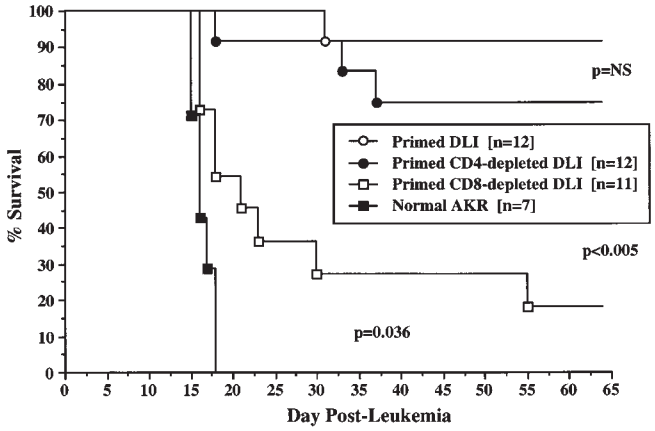

Figure 6. CD8$^+$ (CD4-depleted) T cells from primed donors mediate a GVL effect in MHC-matched chimeras given DLI

Irradiated AKR recipients were given 10^7 T-depleted B10.BR BM cells. Twenty-one days after marrow transplant, the recipients were given DLI with 3×10^7 B10.BR spleen cells (untreated, CD4-depleted, or CD8-depleted as in Fig. 4) from donors preimmunized with AKR alloantigens (see MATERIALS AND METHODS). One week later (28 days post-BMT), the mice were challenged with an i.v. dose of 1000 AKR-M2 leukemia cells. Normal, nontransplanted AKR mice were also injected with leukemia. The survival curves were derived from the combined results of two experiments.

that of mice given nondepleted primed splenocytes (92%). CD8 depletion of primed donor splenocytes (CD4$^+$) significantly reduced the GVL effect of DLI compared with those given nondepleted splenocytes or CD4-depleted splenocytes ($p < 0.005$) (18% survival); however, the GVL effect was not completely lost. The majority of animals given DLI with T-subset–depleted splenocytes that died after leukemia challenge were found to have splenomegaly, which is indicative of leukemia progression. Overt clinical signs of GVHD (diarrhea, wasting, and ruffled fur) were not observed in mice given DLI with T-subset–depleted primed donor cells.

DISCUSSION

In the current report, we investigated the consequences of increased immunogenetic disparity after DLI in a mouse strain combination with complete MHC mismatch. We had previously shown that DLI could be administered to murine MHC-matched and -haplomismatched allogeneic recipients without GVHD if infusion of the donor cells was delayed until 21 days posttransplant [7,8]. In contrast to our previous data, GVHD occurred in an MHC-mismatched combination when DLI was given 21 days posttransplant (Figs. 1 and 2). This confirms the work of Blazar et al. [15], who reported that GVHD developed after DLI with MHC-mismatched murine donor cells at 21 days posttransplant. We extend their observation by showing that GVHD was avoided if DLI administration was delayed an additional week (day 28 post-BMT) (Fig. 2), indicating that a longer time interval between BMT and DLI was required to avoid GVHD in full MHC-mismatched recipients. It is unknown why this additional week is required. We have evidence, however, that de novo generated thymus-derived cells of donor origin play an important role in the suppression of GVHD after DLI (B.D.J., unpublished observations), and interestingly, preliminary data suggests that the kinetics of "suppressor" cell development is delayed in the MHC-mismatched model compared with the MHC-matched model. This delayed development of suppressor cells may explain the additional week required to avoid GVHD in the MHC-mismatched model. Increased immunogenetic disparity may also be partly responsible for the increased time interval, but we have no evidence to support this possibility.

We previously reported a correlation between low alloreactive T-helper frequencies and absence of GVHD after DLI in an MHC-matched model of DLI [8]. In the present study, donor antihost T-helper cell frequencies in day-21 recipients of MHC-mismatched DLI were comparable to those of the GVHD controls (Table 1), lending further support to the correlation between T-helper frequency and GVHD. In agreement with our mouse data, Bunjes et al. [16] found an association between the presence of alloreactive T-helper cells and GVHD in patients after DLI. In contrast to the T-helper frequency data, CTL frequencies in the spleens of day-21 DLI recipients were significantly lower than those of the GVHD controls (Table 1). We do not know the significance of this finding. However, since GVHD is less severe in recipients of day 21 DLI than in recipients of spleen cells on the day of transplant (Fig. 1, B vs. A), we speculate that the lower CTL frequencies may be a reflection of less severe GVHD in these mice. Perhaps alloreactive T-helper cells in the DLI chimeras provide less efficient "help" than those in the GVHD controls, thereby resulting in the lower CTL frequencies found in the spleens of DLI recipients. Decreased T-helper efficiency in the DLI-treated mice might be due to lower cytokine production by these helper cells. This possibility will be pursued in future studies.

Although the severity of GVHD after DLI is less than expected considering the high numbers of donor T cells administered, GVHD can still be a significant clinical problem [3,4]. A number of approaches are being used in attempts to reduce the incidence and severity of GVHD after DLI, including suicide gene insertion [17,18], administration of lower or escalating numbers of donor cells [19,20], and selective depletion of T cell subsets [5]. Giralt et al. [5] reported that only three of 10 patients given CD8-depleted DLI for treatment of relapsed CML developed GVHD, suggesting that the incidence of GVHD can be lowered using this approach. In agreement with this clinical observation, DLI with purified CD4$^+$ or CD8$^+$ T cells in our mouse studies significantly reduced the severity of GVHD in MHC-mismatched recipients (Fig. 3 and Table 2).

The effector cells responsible for the GVL effect of DLI are unknown, but T cells and natural killer (NK) cells are considered likely candidates. For CML, CD4$^+$ T cells have been implicated as effectors in the GVL response [5,21,22]. This finding may be partly due to the fact that CML cells express MHC class II molecules [23], which could allow for the direct recognition of tumor cells by CD4$^+$ T cells. T cells were required for the GVL effect in our murine models of DLI (Figs. 4–6). Furthermore, in both the MHC-matched and -mismatched DLI models, CD8$^+$ cells were critical for the GVL response against the

MHC class I^+II^- AKR acute T cell leukemia. $CD8^+$ donor T cells have been found to be important in the GVL effect against two other murine acute leukemias after DLI [15]. One was an acute myeloid tumor (C1498), and the other an acute T cell leukemia/lymphoma (EL4) [15]. Similar to the AKR leukemia, both of these tumors are MHC class I^+II^-. The lack of MHC class II expression may explain why $CD8^+$ cells were the dominant GVL effectors in all three murine DLI models.

Although $CD8^+$ T cells were essential, depletion of $CD4^+$ donor T cells in an MHC-matched DLI model also significantly compromised the GVL effect (Fig. 5). If the donor T cells in this combination were primed to host alloantigens, however, then CD4 depletion did not significantly diminish GVL reactivity (Fig. 6). These data suggest that $CD4^+$ cells play an important role during the afferent phase of the GVL reaction, most likely by providing help to precursor $CD8^+$ effector cells. Evidence to support this suggestion comes from data in the MHC-mismatched model, in which administration of purified $CD8^+$ cells induced a significantly weaker GVL response than the combination of CD4 and CD8 cells together (Fig. 4B and C, Table 3). $CD4^+$ cells may also contribute to the effector phase of the GVL response, since CD8 depletion in the experiment shown in Fig. 6 did not completely eliminate antitumor reactivity. These data do not rule out the possibility that the remaining GVL reactivity was mediated by other cells such as NK cells or a small population of contaminating $CD8^+$ T cells that survived the complement treatment. When MHC-mismatched chimeras were challenged with a low dose of leukemia, purified CD4 cells were capable of inducing a modest GVL effect (Fig. 4A). The GVL effect was not observed when the DLI-treated recipients were challenged with higher doses of leukemia (Fig. 4B and C). Thus, purified $CD4^+$ donor cells alone were capable of inducing an antitumor response against the AKR acute leukemia, but the response was relatively weak.

In the MHC-matched model, DLI with unseparated donor spleen cells primed to host alloantigens did not induce clinically evident GVHD in the GVL experiments shown in Fig. 6. These results are different from our previous data, in which MHC-matched recipients given DLI with primed cells were at risk for developing chronic GVHD [8]. We do not know the reason for these disparate results, but we hypothesize that one or more of the following factors may have been influential. First, since the GVHD is chronic in nature [8], we may not have followed the animals long enough to observe the development of clinically evident GVHD. Second, the leukemia challenge may have somehow had an impact on the development of GVHD. Previous studies have shown that mice challenged with leukemia develop less GVHD than their unchallenged counterparts (R.L.T., unpublished observations). Last, the AKR hosts in these experiments were conditioned for BMT with a higher dose of TBI than was used in our previous studies (1100 vs. 900 cGy). Shlomchik et al. [24] have presented mouse data that suggests host antigen-presenting cells (APCs) are required for induction of GVHD. If the higher dose of TBI used in the current experiments resulted in decreased levels of host-type APCs at the time of DLI, it might explain the absence of GVHD with primed DLI. Unfortunately, we have no techniques available to investigate whether there were decreased levels of host APCs in these chimeras at the time of DLI.

Biological properties of the leukemia may have a major impact on which T cell subsets are needed for a GVL effect after DLI. Levels of MHC antigen expression, costimulatory molecule expression, tumor-specific antigen expression, and kinetics of tumor growth are some of the factors that may determine the relative roles of T-subsets. It is becoming clear that $CD4^+$ cells are important for the GVL effect against CML [5,21,22], and selective depletion of donor CD8 cells for DLI has not compromised the antitumor response in patients with relapsed CML [5]. This may have to do with the fact that CML cells often express MHC class II antigens. Which T cells are important in GVL reactions against the acute leukemias is unknown. We have chosen the highly aggressive AKR T cell leukemia as a model of human acute leukemia. Since DLI has been less effective for the treatment of acute leukemias, compared with CML, we hope this model may be relevant for investigating ways of enhancing the GVL effect of DLI. Based on our results indicating that $CD8^+$ donor T cells are critical to the GVL effect of DLI against the AKR acute leukemia, we are focusing our efforts on enhancement of the CD8-mediated GVL effect without increasing GVH reactivity.

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